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The *firA* Gene of *Escherichia coli* Encodes UDP-3-*O*-(*R*-3-hydroxymyristoyl)-glucosamine *N*-Acyltransferase

THE THIRD STEP OF ENDOTOXIN BIOSYNTHESIS*

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The possibility that the *firA* gene of *Escherichia coli* (Dicker, I. B., and Seetharam, S. (1991) *Mol. Microbiol.* 6, 817–823) might function in lipid A biosynthesis was examined based on its homology to the *lpxA* gene, which encodes UDP-*N*-acetylglucosamine *O*-acyltransferase, the first enzyme in lipid A formation. Extracts of a temperature-sensitive *firA* mutant, RL-25, were assayed for their ability to acylate UDP-GlcNAc, using a coupled assay. The results suggested that extracts of RL-25 might be defective in the third enzyme of this pathway, the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase. Living cells of RL-25 also displayed a 5-fold decreased rate of lipid A biosynthesis at the nonpermissive temperature as judged by a $^{32}P_i$ incorporation assay. In order to examine *N*-acyltransferase activity directly, the substrate [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN was synthesized enzymatically. *N*-Acyltransferase specific activity in RL-25 extracts was reduced to less than 10% of wild-type. When the wild-type *firA* gene was cloned into a T7-based expression vector, *N*-acyltransferase specific activity increased almost 360-fold relative to wild-type extracts, demonstrating that *firA* is the structural gene for the enzyme. The *N*-acyltransferase displays absolute specificity for the *R*-3-OH moiety of *R*-3-hydroxymyristoyl-ACP, as does the *O*-acyltransferase, consistent with the placement of *R*-3-hydroxymyristate in *E. coli* lipid A.

The lipid A component of lipopolysaccharide resides in the outer leaflet of the outer membrane of Gram-negative bacteria and is the causative agent of endotoxin-induced shock (1). The structure of lipid A found in *Escherichia coli* and *Salmonella typhimurium* consists of a β,1–6 linked glucosamine disaccharide acylated with *R*-3-hydroxymyristate at positions 2, 3, 2′, and 3′. Myristate and laurate are esterified to the two *R*-3-hydroxy groups of the nonreducing glucosamine *R*-3-hydroxymyristate moieties, and positions 1 and 4′ are phosphorylated (2).

The biosynthesis of *E. coli* lipid A has been elucidated (2). Although some of the enzymes involved have been purified and several of the relevant genes are known, not all of the genes involved in the pathway have been described (2). Among the known genes are *lpxA* and *lpxB*, which are located at minute 4 of the *E. coli* chromosome and encode UDP-GlcNAc *O*-acyltransferase (Fig. 1) and lipid A disaccharide synthase (not shown), respectively (3). The *envA* gene at minute 2 has recently been found to encode the second enzyme in the pathway, UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase (4) (Fig. 1). The gene for 3-deoxy-D-*manno*-octulosonate transferase (not shown), *kdtA*, resides at minute 81 (5, 6). Among the unknown genes is that encoding UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase, which catalyzes the third step in the pathway (Fig. 1).

A gene known as *firA*, located at minute 4 of the *E. coli* chromosome, is part of the same complex operon as *lpxA* and *lpxB* (7, 8). From the DNA sequence of *firA*, one predicts a 36-kDa protein that has significant homology to *lpxA* (7–11). *firA* is essential for the growth of *E. coli* (8, 11). It was originally described as a mutation that eliminates rifampicin resistance associated with mutations of the *rpoB* gene (8, 11, 12). The latter encodes a subunit of RNA polymerase. The temperature-sensitive *firA200* mutation also renders RNA synthesis somewhat thermosensitive (8, 11, 12). For these reasons, *firA* was postulated to regulate the transcription apparatus (8, 11). However, it is not a subunit of RNA polymerase.

Mutations in lipid A biosynthesis are known to confer rifampicin hypersensitivity (13) and inhibit cell growth (2), much like the phenotypes associated with mutations in *firA*. Based on the proximity of *firA* to other lipid A biosynthetic genes and its sequence homology to *lpxA*, we decided to examine whether or not *firA* encodes UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase. We now demonstrate that the mutant *firA* allele (*firA200*) confers a defect in lipid A synthesis in cells at 43 °C, that extracts of cells bearing *firA200* possess a defective thermolabile UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase, and that overexpression of the wild-type *firA* gene leads to massive overproduction of *N*-acyltransferase. Thus, *firA* is an essential gene encoding an enzyme required for lipid A biosynthesis, and its role in the regulation of RNA polymerase must be questioned.

EXPERIMENTAL PROCEDURES

Materials—$^{32}P_i$ was obtained from Du Pont-New England Nuclear and [α-^{32}P]ATP was purchased from Amersham Corp. Taq polymerase was from Perkin-Elmer Cetus. Inorganic pyrophosphatase and UDP-glucose pyrophosphorylase were from Sigma. Silica gel 60 thin layer plates (0.25 mm) and PEI[1]-cellulose F thin layer plates (0.1 mm) were the products of E. Merck, Darmstadt, Germany. *E. coli* K12 cells were purchased from Grain Processing, Muscatine, IA.

Bacterial Strains—Bacterial strains and plasmids used in this study are listed in Table I. Strains R477, RL-25, and NM554 are derivatives

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[1] The abbreviations used are: PEI, polyethyleneimine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ACP, acyl carrier protein; BSA, bovine serum albumin; Pipes, 1,4-piperazinediethanesulfonic acid; HPLC, high performance liquid chromatography.

TABLE I
Bacterial strains

Strain	Relevant genotype	Source
R477	*F⁻ rpsL136 eda his-4 leu-6 thr-1*	J. Adler[a]
RL-25	*Hfr fhuA67 firA200(Ts) relA spoT1 metB1*	CGSC[b]
NM554	*F⁻ recA13 araD139 Δ(ara-leu)7696 Δ(lac)17A galU galK hsdR rpsL mcrA mcrB*	Stratagene
BL21(DE3)	*F⁻ ompT* (r_B- m_B-)	F. Studier[c]

[a] University of Wisconsin, Madison, WI.
[b] *E. coli* Genetic Stock Center, Yale University, New Haven, CT.
[c] Brookhaven National Laboratory, Upton, NY.

of *E. coli* K12. Strain BL21(DE3) is a derivative of *E. coli* B. Strain RL-25 (CGSC 7009) was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT.

Preparation of Cell Extracts—Unless otherwise stated, cell-free crude extracts were prepared by growing a culture in LB broth (3) at 37 °C to late log phase. Ampicillin (100 μg/ml) and/or chloramphenicol (20 μg/ml) were added when appropriate. Cells were harvested by centrifugation at 8,000 × *g* for 10 min and washed once in 1 volume of 50 mM potassium phosphate, pH 7.0. Cells were resuspended in the same buffer (1–10 mg/ml final protein concentration) and broken in a French pressure cell at 18,000 p.s.i. Debris and unbroken cells were removed by centrifugation at 8,000 × *g* for 10 min. Protein concentrations were determined by the method of Smith *et al.* (14).

Construction of BL21(DE3)/pLysS/pTK106—The *firA* gene product was overproduced using the T7 expression system of Studier *et al.* (15). The *firA* gene was amplified by the polymerase chain reaction using Taq polymerase and R477 genomic DNA as the template. The upstream primer contained a G/C clamp, the vector sequence starting with an *Xba*I site through the initiation site of T7 transcription, and the translation initiation site of the *firA* gene. It had the following sequence: 5′-CGCGCGTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCCTTCAATTCGACTGGCTG-3′. The downstream primer contained a G/C clamp, a *Bam*HI restriction site, and the *firA* anticoding strand which included two stop codons. The downstream primer had the following sequence: 5′-GCGCGCGGATCCTTATTAGTCTTGTTGATTAACCTTGCG-3′. The major PCR product (1.1 kilobase pairs) was digested with *Xba*I and *Bam*HI and then ligated into a similarly digested pET11a vector (15). Transformation of the ligation reaction into competent NM554 cells prepared by treatment with $CaCl_2$ (16) gave ampicillin-resistant colonies. The pTK106 plasmid, identified as a colony containing a 1.1-kilobase pair insert, was isolated by an alkaline lysis procedure (17) and transformed into competent BL21(DE3)/pLysS cells. Colonies resistant to ampicillin and chloramphenicol were selected.

Overexpression of the FirA Gene Product—Cultures of BL21(DE3)/pLysS/pTK106 were grown at 37 °C to an optical density at 570 nm of approximately 0.4, at which time IPTG was added to a final concentration of 1 mM. After 30 min, rifampicin (final concentration = 200 μg/ml) was added, and the incubation was continued for another 2 h. Extracts were then prepared as described above.

Polyacrylamide Gel Electrophoresis—The overexpression and relative molecular mass of the *firA* protein were determined by SDS-polyacrylamide gel electrophoresis. The separating and stacking gels were 15 and 5% polyacrylamide, respectively. Electrophoresis was carried out at approximately 35 mA in the discontinuous system of Laemmli (18).

Lipid A Analysis—The assay for lipid A biosynthesis in living cells was carried out as described by Galloway and Raetz (19). Pulse labeling with $^{32}P_i$ (30 μCi/ml) was carried out in G-56 medium either during exponential growth at 30 °C or following a 1.5-h temperature shift to 43 °C.

Synthesis of R-3-Hydroxymyristoyl-Acyl Carrier Protein—The substrate *R*-3-hydroxymyristoyl-ACP was prepared from purified ACP and synthetic *R*-3-hydroxymyristate, using a preparation of *E. coli* acyl-ACP synthetase that had been immobilized on blue-Sepharose.

The ACP was purified by a modification of the procedure of Rock and Cronan (20). *E. coli* K12 cells (1,000 g) were thawed at 4 °C and suspended in 100 ml of 1 M Tris-HCl, pH 8.0, containing 1 M glycine and 0.25 M EDTA. The mixture was stirred well, and 900 ml water was added, followed by 30 mg of lysozyme dissolved in 5 ml of water. The solution was stirred for 90 min, and an equal volume of 0.5% Triton X-100 (w/v) was added. The mixture was homogenized in a Waring blender and stirred for 1 h. An equal volume of isopropanol was slowly added and stirred for 90 min. The solution was centrifuged at 8,000 × *g* for 20 min. The supernatant was removed and added to 500 g of DEAE-cellulose (Whatman DE52), which had been equilibrated with 10 mM sodium phosphate, pH 6.5. The slurry was mixed gently by shaking overnight at 4 °C. This suspension was collected on a Buchner funnel and washed with 5 liters of 10 mM Pipes, pH 6.1, containing 0.15 M LiCl and 0.1% Triton X-100, followed by a second wash with 5 liters of 10 mM Pipes, pH 6.1, containing 0.15 M LiCl. The ACP was eluted from the resin in 400 ml fractions with 0.5 M LiCl in 10 mM Pipes, pH 6.1. The ACP was recovered in fractions 2 and 3, which were brown in color. These fractions were acidified to pH 3.9 with glacial acetic acid and held at 4 °C for 60 h. The mixture was then centrifuged at 10,000 × *g* for 30 min. In our hands, the ACP product was recovered in the supernatant, the pH of which was adjusted to 7.0 with 5 M NaOH, followed by concentration to about 50 ml at 4 °C using an ultrafiltration apparatus. The ACP solution was about 85% pure, as judged by gel electrophoresis (21), and it was stored at −20 °C.

To obtain acyl-ACP synthetase (21, 22), *E. coli* K12 cells (200 g) were thawed and suspended in 450 ml of 50 mM Tris-HCl, pH 8.0. All operations were carried out at 0–4 °C. DNase II (5 mg) was dissolved in 1 ml of water and added to the cells. The cells were broken by one pass through a French Pressure cell at 18,000 p.s.i. The solution was centrifuged at 15,000 × *g* for 20 min. The supernatant was removed and adjusted to 10 mM $MgCl_2$ with a 1 M stock solution. The solution was then ultracentrifuged at 50,000 × *g* for 90 min. The pellet was resuspended in 120 ml of 50 mM Tris-HCl, pH 8.0, with a Potter-Elvehjem homogenizer. To this suspension was added 120 ml of 50 mM Tris-HCl, pH 8.0, containing 1 M NaCl and 20 mM $MgCl_2$. The mixture was stirred for 15 min and ultracentrifuged at 50,000 × *g* for 90 min. The pellet was resuspended in 80 ml of 50 mM Tris-HCl, pH 8.0, and homogenized again. To the suspension was added 80 ml of 50 mM Tris-HCl, pH 8.0, containing 4% Triton X-100 and 20 mM $MgCl_2$. The mixture was stirred for 30 min and ultracentrifuged at 50,000 × *g* for 90 min. The supernatant was removed, made 5 mM in ATP, and transferred to a flask containing a suspension of blue-Sepharose equilibrated overnight with 50 mM Tris-HCl, pH 8.0, and 2% Triton X-100. (The latter was prepared by hydrating 12 g of blue-Sepharose with 250 ml of water, followed by 250 ml of 50 mM Tris-HCl, pH 8.0, and 2% Triton X-100. Excess buffer was decanted prior to adding the membrane extract to the settled suspension of blue-Sepharose). The mixture was swirled at 4 °C for 36 h. The blue-Sepharose was then washed on a Buchner funnel with: 1) 400 ml of 50 mM Tris-HCl, pH 8.0, containing 2% Triton X-100; 2) 400 ml of 50 mM Tris-HCl, pH 8.0, containing 2% Triton X-100 and 0.6 M NaCl; and 3) 400 ml of 50 mM Tris-HCl, pH 8.0, containing 2% Triton X-100. Near the end of the last wash a small amount of buffer solution was left standing above the blue-Sepharose. Catalytically active acyl-ACP synthase was partially purified in this manner, but it remained firmly bound to the blue-Sepharose. The immobilized enzyme was used immediately or within a few days of preparation; short term storage was at 4 °C.

The enzymatic acylation of the purified ACP with *R*-3-hydroxymyristate was carried out as follows. ACP (104 mg) and 8.6 mM dithiothreitol were incubated in 50 ml of 40 mM Tris-HCl, pH 8.0, in a sealed tube at 37 °C for 1 h. A mixture consisting of 0.7 M LiCl, 40 mM $MgCl_2$, 20 mM ATP, pH 8, 750 μM *R*-3-hydroxymyristate ephedrine salt (23), and 2.7% Triton X-100 in 32 ml of 540 mM Tris-HCl, pH 8.0, was added to the tube containing the ACP. This mixture was then added to the 40 ml of the blue-Sepharose suspension bearing the immobilized synthase (from which the final supernatant described above had been decanted), and the reaction was mixed gently for 20 h at 4 °C. The extent of acylation was determined by letting the resin settle and analyzing 5 μl portions of the supernatant on a 17%

nondenaturing polyacrylamide gel (21). Under these conditions, the acylated ACP migrates faster than the unacylated ACP. Acylation is essentially quantitative.

To isolate the product, the blue-Sepharose suspension was poured into a column, and the run-through was collected. The column was further washed with 160 ml of 10 mM Tris-HCl, pH 8.0, containing 0.2% Triton X-100, and the run-through and wash were combined. The sample was diluted 10-fold with water and mixed overnight at 4 °C with 120 ml (settled volume) of fast flow DEAE-Sepharose, equilibrated with 10 mM bis-tris, pH 6.0. The mixture was poured into a large Buchner funnel, and the DEAE-Sepharose was washed as follows: 1) 1 liter of 10 mM bis-tris, pH 6.0; 2) 1 liter of 10 mM bis-tris, pH 6.0, containing 50% isopropanol (v/v); and 3) 1 liter of 10 mM bis-tris, pH 6.0. Elution of the product was carried out by transferring the DEAE-Sepharose to a column and washing with 2 bed volumes of 10 mM bis-tris, pH 6.0, containing 0.6 M LiCl. Fractions of 30 ml were collected. The *R*-3-hydroxymyristoyl-ACP was in fractions 4 and 5, which had a yellow color. These fractions were combined and dialyzed against 10 liters of 0.5 mM bis-tris, pH 6.0, followed by dialysis against 10 liters of 0.1 mM bis-tris, pH 6.0. The sample was then lyophilized to dryness and dissolved in 5 ml of water. Aliquots stored at −80 °C were stable for several years. The sample was 90% pure, as judged by electrophoresis on a 17% nondenaturing polyacrylamide gel (21).

Synthesis of [α-^{32}P]UDP-GlcNAc—[α-^{32}P]UTP (1 mCi, 800 Ci/mmol) was allowed to react at room temperature for 16 h in a 250-μl reaction mixture consisting of 0.5 mM glucosamine 1-phosphate, 1 mM $MgCl_2$, 5 mM dithiothreitol, 100 mM Tris-HCl, pH 8.0, and 50 units each of inorganic pyrophosphatase and UDP-glucose pyrophosphorylase. At the end of this incubation, the mixture was acetylated with acetic anhydride by the addition of an equal volume of deionized water, 300 μl of methanol, 10 μl of saturated sodium bicarbonate, and 10 μl of acetic anhydride. The reaction was allowed to proceed for 3 min at room temperature and was then quenched by placement in a boiling water bath for 90 s to destroy excess acetic anhydride. The crude mixture was diluted with 7 ml of deionized water and loaded onto a 5-ml column of DEAE-cellulose (pre-equilibrated with 10 mM triethylammonium bicarbonate, pH 7.0). The column was washed free of salts with 30 ml of water, followed by elution of the product with a 20-ml wash of 100 mM triethylammonium bicarbonate, pH 7.0. The eluate was collected in a 50-ml plastic tube and lyophilized. The product was redissolved in 800 μl of water, and the pH was adjusted to 7.0 with 1 M NaOH.

Synthesis of [α-^{32}P]UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc—Radiolabeled and carrier materials were produced separately. Carrier material was made using a very low specific radioactivity of [α-^{32}P]UDP-GlcNAc (3 mCi/mmol), which was used to quantitate the yield and concentration of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc during its preparation, but this amount of radioactivity did not contribute significantly to the specific radioactivity of the substrate in the standard assay mixture.

High specific radioactivity [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc was produced from a reaction mixture containing [α-^{32}P]UDP-GlcNAc (100 μCi, 0.18 μM), 18 μM *R*-3-hydroxymyristoyl-ACP, 10 mg/ml fatty acid-free BSA, 40 mM Hepes, pH 8.0, and purified UDP-GlcNAc *O*-acyltransferase (0.2 μM) (48) in a final volume of 688 μl. Low specific radioactivity (high mass) [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc was produced from a reaction mixture identical to that above, but containing [α-^{32}P]UDP-GlcNAc (1 μCi, 480 μM) and *R*-3-hydroxymyristoyl-ACP (480 μM). Reactions were initiated by the addition of enzyme and were performed at 30 °C for 1 h. The entire reaction mixture was loaded onto a 10-ml column of DEAE-Sepharose fast flow column (prewashed with 30 ml of 50 mM bis-tris, pH 6.0, containing 1 M NaCl, followed by 40 ml of 10 mM bis-tris, pH 6.0), which was connected to a Sep-Pak C_{18} cartridge (Waters). The Sep-Pak C_{18} cartridge had been washed with 10 ml of methanol followed by 20 ml of deionized water. After loading the sample, the combined column and cartridge were washed with 30 ml of 200 mM sodium chloride. The Sep-Pak, which contained the desired product, was removed and further washed with 30 ml of water. The UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc was eluted from the cartridge with methanol in 0.75-ml fractions. The most highly radioactive fractions (usually fractions 2–4) were combined, dried in a Speed-Vac, and redissolved in 10 mM bis-tris, pH 5.5, to give a final concentration of 0.5–1.0 mM. The product was stored at −20 °C.

Synthesis of [α-^{32}P]UDP-3-O-(R-3-hydroxymyristoyl)-GlcN—Radiolabeled UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN can be prepared with membrane-free crude extracts of wild-type cells, as described previously (25). However, a new method was developed to remove the acetate moiety using partially purified UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase (4), the second enzyme in the pathway (Fig. 1). A typical reaction mixture contained [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (10 μCi, 30 μM), 1 mg/ml BSA, 50 mM bis-tris, pH 5.5, and UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase (~0.6 μM) in a final volume of 50 μl. The reaction was incubated at 30 °C for 5 min. The final product was purified by HPLC on a Zorbax ODS column (4.6 mm × 25 mm, Du Pont) using a 10-min gradient from 70:30 to 55:45 of 0.1% ammonium acetate, pH 5.4:acetonitrile (v/v) with a flow rate of 1 ml/min. Most of the radioactivity was associated with elution of the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN product at 7.5–8.2 min. Residual UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc eluted at 6.0–6.5 min. The HPLC-purified material was lyophilized, redissolved in 10 mM bis-tris, pH 5.5, at a concentration of 2–10 μM, and stored at −20 °C.

Coupled Assay for the First Three Enzymes of Lipid A Biosynthesis—A coupled assay for observing the flow of metabolites derived from radiolabeled UDP-GlcNAc in the early steps of lipid A biosynthesis (Fig. 1) was based on the work of Anderson and Raetz (22, 26). Assay mixtures contained [α-^{32}P]UDP-GlcNAc (0.5 μCi, 200 μM), 50 μM *R*-3-hydroxymyristoyl-ACP, *E. coli* extract as indicated, and 40 mM Hepes, pH 8.0, in a final volume of 20 μl. After 5 min at 30 °C, 5-μl portions of the reaction mixtures were spotted directly onto silica plates. The spots were thoroughly dried, and the plates were developed in chloroform:methanol:water:acetic acid (25:15:4:2, v/v), followed by overnight autoradiography. The products were scraped from the plates and quantitated by liquid scintillation spectrometry. UDP-GlcNAc, monoacylated sugar nucleotides, and UDP-diacyl-GlcN are separated by this procedure; formation of the latter requires the proper functioning of all three of the enzymes shown in Fig. 1.

UDP-3-O-(R-3-hydroxymyristoyl)-GlcN N-Acyltransferase Assays—A more direct and specific measure of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase activity was developed using purified [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN as the substrate. Unless otherwise stated, assay mixtures contained [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN (0.01 - 0.05 μCi, 1 μM), 1 μM *R*-3-hydroxymyristoyl-ACP, 1 mg/ml BSA, *E. coli* extract as indicated, and 50 mM Hepes, pH 8.0, in a final volume of 10 μl. After 5 min at 30 °C, 5-μl portions of the reaction mixtures were spotted directly onto silica plates. After drying, the plates were developed in chloroform:methanol:water:acetic acid (25:15:4:2, v/v). Following overnight autoradiography, the products were scraped from the plates and quantitated by liquid scintillation spectrometry. A unit of enzymatic activity is that amount capable of generating 1 nmol/min of product under standard assay conditions.

In initial experiments, small amounts of diacylated product were observed in some control reactions lacking enzyme (data not shown) with certain lots of *R*-3-hydroxymyristoyl-ACP. This anomaly was due to contamination of the *R*-3-hydroxymyristoyl-ACP with small amounts of *N*-acyltransferase, which could be inactivated by preincubation of the *R*-3-hydroxymyristoyl-ACP substrate at 80 °C for 20 min. All subsequent assays were carried out with heat-treated ACP.

RESULTS

Strain RL-25 (firA200) Is Defective in Lipid A Biosynthesis—The *firA* gene has significant homology to the *lpxA* gene, known to encode the first acyltransferase in lipid A biosynthesis (Fig. 1) (3, 7–11). Additionally, the *firA* gene is part of the same complex operon as *lpxA* (8, 11).

In order to examine whether or not *firA* plays a role in lipid A biosynthesis, cells of *E. coli* strain RL-25, which carries a temperature-sensitive mutation in *firA*, were assayed for their ability to synthesize lipid A at 30 and 43 °C. We employed the method of Galloway and Raetz (19), which is based on the measurement of $^{32}P_i$ incorporation into the 4′ position of lipid A relative to glycerophospholipids. At the nonpermissive temperature (43 °C), the *firA200* mutation caused a dramatic reduction in the rate of lipid A biosynthesis during a 5-min pulse labeling. As seen in Fig. 2, the ratio of the rates of lipid A to glycerophospholipid synthesis was reduced 5-fold at 43 °C compared with 30 °C. R477, a *firA*$^+$ *E. coli* K12 control strain, displayed a constant ratio (0.096) at these temperatures, consistent with earlier findings (19).

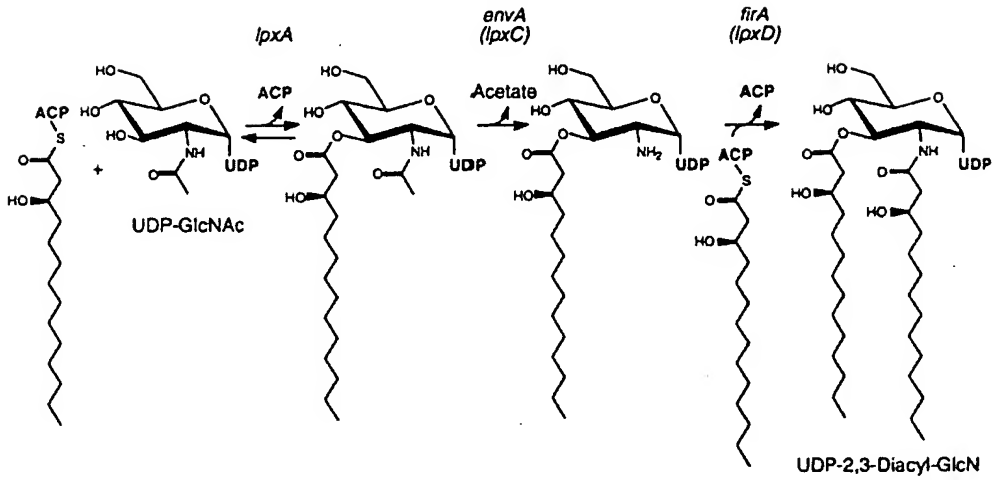

FIG. 1. **The first three steps of lipid A biosynthesis in *E. coli*.** *R*-3-hydroxymyristoyl acyl carrier protein (*ACP*) is an intermediate in fatty acid synthesis (1, 2). If it is not used for the generation of lipid A, it can be elongated to palmitate. The further reactions involved in the conversion of UDP-2,3-diacyl-GlcN to lipid A have been reviewed elsewhere (1, 2). Genetic symbols indicate the structural genes encoding the corresponding enzyme. We suggest that *envA* and *firA* be designated *lpxC* and *lpxD*, repectively.

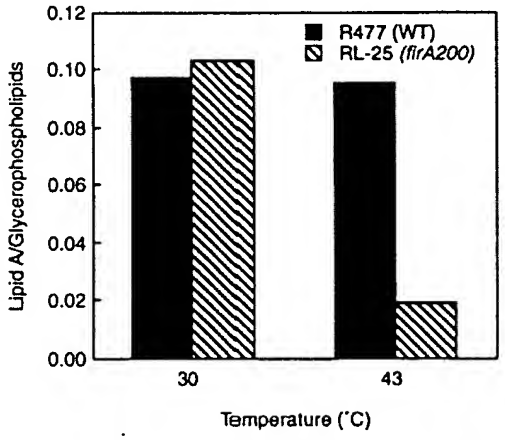

FIG. 2. **Defective lipid A biosynthesis in cells of mutant RL-25 (*firA200*) at 43 °C.** Strains R477 (*firA*$^+$) and RL-25 (*firA200*) were grown in G-56 medium at 30 °C to A_{570} of approximately 0.2. The samples were then either pulse-labeled with $^{32}P_i$ for 5 min at 30 °C or temperature-shifted to 43 °C for 90 min before a 5-min pulse labeling. The results are expressed as the ratio of the rates of lipid A to phospholipid synthesis (19).

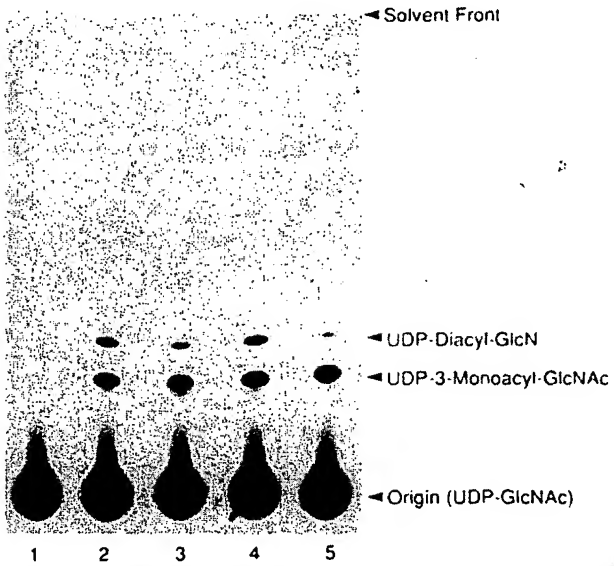

FIG. 3. **Defective formation of [α-^{32}P]UDP-diacyl-GlcN from [α-^{32}P]UDP-GlcNAc in extracts of mutant RL-25.** This coupled assay for the first three enzymes of lipid A biosynthesis (22, 26) was carried out with 1 mg/ml of extract, as described under "Experimental Procedures." *Lane 1*, no enzyme control. *Lane 2*, wild-type extract (R477) held for 10 min at 30 °C, then assayed for 5 min at 30 °C. *Lane 3*, mutant extract (RL-25) held for 10 min at 30 °C, then assayed for 5 min at 30 °C. *Lane 4*, wild-type extract (R477) held for 10 min at 43 °C, then assayed for 5 min at 30 °C. *Lane 5*, mutant extract (RL-25) held for 10 min at 43 °C, then assayed for 5 min at 30 °C.

O-Acylation of UDP-GlcNAc in RL-25 (firA200) Is Unaffected—Crude extracts of *E. coli* are capable of converting UDP-GlcNAc to more hydrophobic metabolites in the presence of *R*-3-hydroxymyristoyl-ACP (22, 26). As can be seen in Fig. 3, two species, [α-^{32}P]UDP-monoacyl-GlcNAc and [α-^{32}P]UDP-diacyl-GlcN are generated from [α-^{32}P]UDP-GlcNAc and *R*-3-hydroxymyristoyl-ACP with extracts of wild-type R477. However, extracts of mutant RL-25 showed diminished ability to form the diacylated species, especially if first preincubated at the nonpermissive temperature (43 °C) for 10 min prior to a 5-min assay at 30 °C. Formation of [α-^{32}P]UDP-monoacyl-GlcNAc was unaffected, demonstrating that the *firA200* mutation impairs either the second or the third enzyme of the pathway (Fig. 1). The mutation cannot be in the structural gene encoding the deacetylase, however, because the *envA* gene at minute 2 encodes the deacetylase, whereas the *firA200* mutation maps at minute 4 (4, 8). Therefore, the *firA200* mutation must affect the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase specifically.

Extracts of RL-25 (firA200) Accumulate [α-^{32}P]UDP-3-O-(R-3-hydroxymyristoyl)-GlcN when Incubated with [α-^{32}P]UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc and R-3-hydroxymyristoyl-ACP—Mild base hydrolysis of mixtures of [α-^{32}P]UDP-3-*O*-acyl-GlcNAc and [α-^{32}P]UDP-3-*O*-acyl-GlcN results in the removal of the *O*-linked acyl groups, allowing the products, [α-^{32}P]UDP-GlcNAc and [α-^{32}P]UDP-GlcN, to be separated by chromatography on PEI-cellulose plates in 0.2

M guanidine HCl (Fig. 4). This procedure can be used to evaluate the proper functioning of the deacetylase (Fig. 1). When a mixture of [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc and *R*-3-hydroxymyristoyl-ACP is incubated with an extract of R477, followed by mild base hydrolysis (0.2 M NaOH), PEI-cellulose analysis revealed [α-^{32}P]UDP-GlcNAc derived from unreacted substrate as the major species (Fig. 4, *lanes 2* and *4*). (In addition, a streak designated "A," previously shown to consist of [α-^{32}P]UDP-2-*N*-(*R*-3-hydroxymyristoyl)-GlcN derived from [α-^{32}P]UDP-diacyl-GlcN (25), was observed, and [α-^{32}P]UMP was formed from [α-^{32}P]UDP-diacyl-GlcN by the action of pyrophosphatases) (25, 26). By comparison, a similar incubation using RL-25 extracts accumulated a significant amount of the deacetylated intermediate (Fig. 1), as judged by the appearance of [α-^{32}P]UDP-GlcN in addition to [α-^{32}P]UDP-GlcNAc (*lanes 3* and *5*). This result provides direct evidence that the deacetylase activity of RL-25 is functional and strongly suggests that the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase activity is deficient. If deacetylation were deficient in this coupled assay system, then the buildup of [α-^{32}P]UDP-3-*O*-acyl-GlcN could not occur, and any deacetylated species produced would be efficiently *N*-acylated, as it is in the wild-type extracts. The reduced formation of [α-^{32}P]UDP-diacyl-GlcN (region A) and [α-^{32}P]UMP in the incubations with RL-25 extracts (Fig. 4) is also consistent with a lesion in the *N*-acyltransferase (Fig. 1). Lastly, a direct assay of the deacetylase (25) revealed only minor differences between wild-type and mutant extracts (Table II).

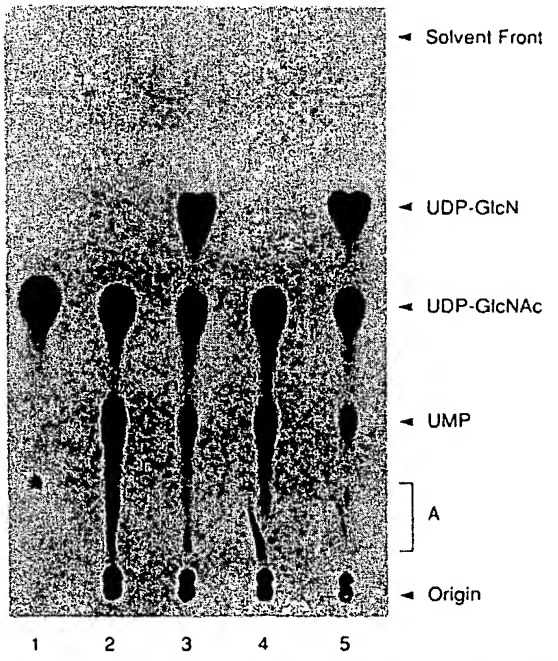

FIG. 4. **Accumulation of [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN in extracts of mutant RL-25 incubated with [α-^{32}P]UDP-3-O-(*R*-3-hydroxymyristoyl)-GlcNAc and *R*-3-hydroxymyristoyl-ACP.** Extracts (1.25 mg/ml, 8 μl) were held at 30 or 43 °C for 10 min, as indicated, and then assayed by adding 2 μl of a mixture containing [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (0.01 μCi, 5 μM) and *R*-3-hydroxymyristoyl-ACP (5 μM) in 50 mM Hepes, pH 8.0. After 5 min at 30 °C, 5-μl portions of the reaction mixtures were then hydrolyzed with 1 μl of 1.25 M NaOH at 30 °C for 10 min. Following neutralization with 1 μl of 1.25 M acetic acid, protein precipitation with 1 μl of 5% trichloroacetic acid, and brief centrifugation, 2-μl portions of the supernatants were spotted onto a PEI-cellulose plate and developed in 0.2 M guanidine HCl. *Lane 1*, no enzyme control. *Lane 2*, wild-type extract (R477), preincubated at 30 °C. *Lane 3*, mutant extract (RL-25), preincubated at 30 °C. *Lane 4*, wild-type extract (R477), preincubated at 43 °C. *Lane 5*, mutant extract (RL-25), preincubated at 43 °C.

A Quantitative Assay for UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine N-Acyltransferase—A quantitative measure of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase activity necessitated the synthesis of the substrate [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN. A two-step enzymatic process was developed using purified UDP-GlcNAc *O*-acyltransferase (48) and partially purified UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc deacetylase (4), as described under "Experimental Procedures." The product was purified by HPLC and was stable for a few weeks when stored at −20 °C.

As shown in Fig. 5*A*, [α-^{32}P]UDP-diacyl-GlcN formation from [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN and *R*-3-hydroxymyristoyl-ACP was proportional to protein concentrations up to 50 μg/ml, when dilutions of R477 extract were used as the enzyme source. Loss of linearity at higher protein concentrations may be caused by further metabolism of [α-^{32}P]UDP-diacyl-GlcN, since the [α-^{32}P] radiolabel is released as [^{32}P]UMP during subsequent steps of the lipid A pathway (22, 26). Fig. 5*B* shows that the *N*-acyltransferase reaction in an R477 extract was linear with time under standard conditions.

With a reliable direct assay for the *N*-acyltransferase, we quantitated the specific activities of both R477 and RL-25 extracts. R477 extracts produced considerably more UDP-diacyl-GlcN than comparable RL-25 extracts, as seen in Fig. 6 (*lanes 2* and *4 versus lanes 3* and *5*). The specific activity for R477 was 1.46 nmol/min/mg, whereas the RL-25 values were decreased by more than 15-fold (Table III). The apparent thermolability of the residual activity in RL-25 extracts supports the view that *firA* is the structural gene encoding the *N*-acyltransferase.

Overexpression of the firA Gene Product—The *firA* gene was amplified by PCR using genomic DNA from strain R477 as a template. The primers for this reaction were designed so that the gene would be flanked by restriction enzyme sites compatible with unidirectional ligation into the pET11a vector of the T7 expression system (15). Following restriction enzyme digestion and ligation of the PCR product and vector, the resulting plasmid pTK106 was transformed into strain

TABLE II

UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc deacetylase activity

The assays were carried out for 5 min at 30 °C following a 10-min preincubation of the extract at the indicated temperatures. Reaction mixtures contained UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc (0.25 μCi, 3 μM), BSA (1 mg/ml), and enzyme extract (1 mg/ml) in 20 μl of 40 mM bis-tris, pH 5.5. After the incubation, 5-μl portions of the reaction mixtures were base hydrolyzed with 1 μl of 1.25 M NaOH at 30 °C for 10 min. Following neutralization with 1 μl of 1.25 M acetic acid and protein precipitation with 1 μl of 5% trichloroacetic acid and centrifugation, 2-μl portions were spotted onto a PEI-cellulose plate and developed in 0.2 M guanidine HCl, as in Fig. 4. Following autoradiography overnight at −70 °C with an enhancing screen, regions containing UDP-GlcN were cut out, and radioactivity was quantitated by liquid scintillation counting.

Strain, preincubation	Specific activity	Relative -fold*
	pmol/min/mg	
R477, 30 °C	72.3	1
RL-25 (*firA200*), 30 °C	54.6	0.76
R477, 43 °C	106.7	1.48
RL-25 (*firA200*), 43 °C	47.7	0.66

* The ~2-fold variation in deacetylase specific activity between R477 and RL-25 is within the range of variation observed with different strains and extracts and is not likely to be significant.

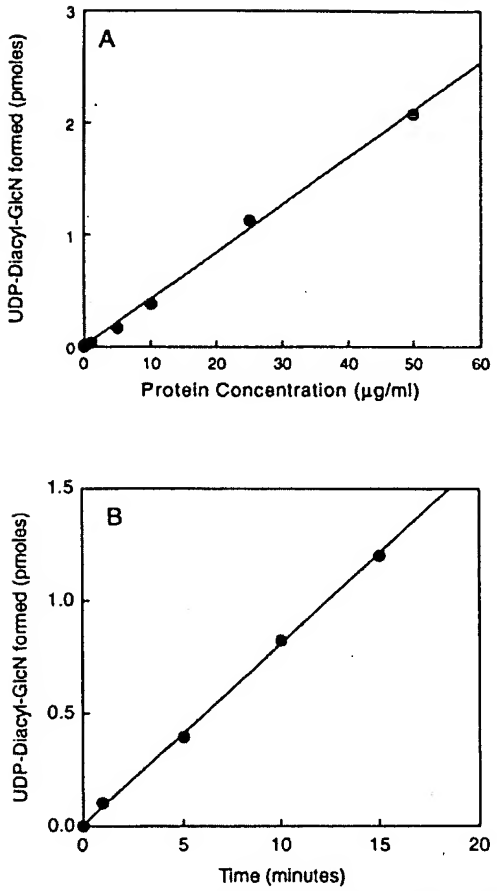

FIG. 5. **Linearity of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase with protein concentration and time.** Assays were carried out as described under "Experimental Procedures" with a wild-type extract made from strain R477. *A*, the incubations were carried out for 5 min. *B*, the concentration of the extract was 5 μg/ml.

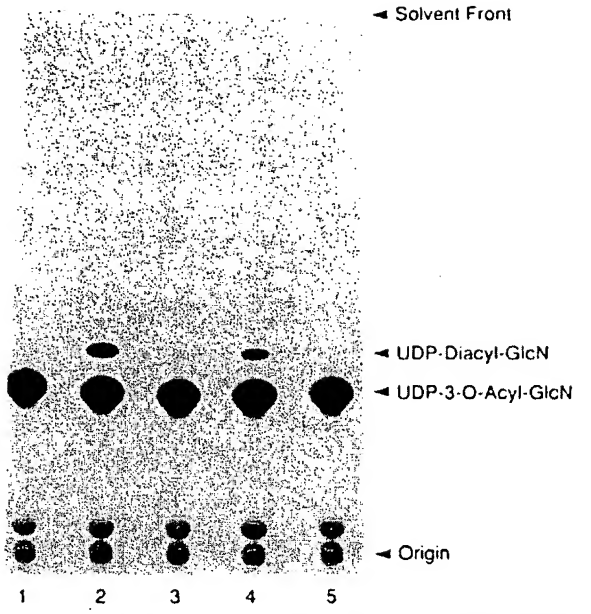

FIG. 6. **Conversion of [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN to [α-^{32}P]UDP-diacyl-GlcN is defective in extracts of mutant RL-25.** The assay conditions for measuring UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferse activity are described under "Experimental Procedures." The protein concentrations in the incubations were 5 μg/ml. *Lane 1*, no enzyme control. *Lane 2*, wild-type extract (R477), held for 10 min at 30 °C, then assayed for 5 min at 30 °C. *Lane 3*, mutant extract (RL-25), held for 10 min at 30 °C, then assayed for 5 min at 30 °C. *Lane 4*, wild-type extract (R477), held for 10 min at 43 °C, then assayed for 5 min at 30 °C. *Lane 5*, mutant extract (RL-25), held for 10 min at 43 °C, then assayed for 5 min at 30 °C.

BL21(DE3)/pLysS. This lysogenic strain contains a gene for T7 RNA polymerase under transcriptional control of tandem lacUV5 promoters. Addition of IPTG induces formation of T7 RNA polymerase, which then transcribes the target DNA (*firA*) in plasmid pTK106 under control of a T7 promoter (15).

Extracts made from IPTG-induced cultures of BL21(DE3)/pLysS/pTK106 initially showed no overexpression of any protein by SDS-PAGE. However, if the induced cultures were treated with rifampicin 30 min after IPTG addition and allowed to incubate for another 2 h, a prominent band at 36 kDa could be visualized (Fig. 7). The BL21 host RNA polymerase is sensitive to inhibition by rifampicin, whereas T7 RNA polymerase is resistant (15). After rifampicin treatment, host mRNAs are not transcribed and preexisting host mRNAs decay, but the mRNAs under the control of the T7 promoter are still produced.

Extracts made from the overproducing *firA*$^+$ strain treated with rifampicin were assayed with [α-^{32}P]UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN in the presence of *R*-3-hydroxymyristoyl-ACP under standard conditions. The specific activities of the extracts were found to be increased approximately 360-fold compared with host and host/vector strains (Table III). Interestingly, the same overexpressing *firA*$^+$ extracts, when assayed with the coupled assay (Fig. 3) using [α-^{32}P]UDP-GlcNAc as the starting substrate (22, 26), did not show increased UDP-diacyl-GlcN formation (data not shown), demonstrating that deacetylation is rate-limiting in the coupled assay (25).

TABLE III

UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine N-acyltransferase activity

In Experiment 1, actual assays were carried out for 5 min at 30 °C following a 10-min preincubation at the indicated temperatures. Reaction mixtures contained 1 μM UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN, 1 μM *R*-3-hydroxymyristoyl-ACP, and 1 mg/ml BSA in 50 mM Hepes, pH 8.0. The R477 and RL-25 extracts were measured at 5 μg/ml protein. The BL21(DE3)/pLysS and BL21(DE3)/pLysS/pET11a extracts were measured at 10 μg/ml, whereas BL21(DE3)/pLysS/pTK106 contained 20 ng/ml protein. In Experiment 2, the extracts were not preincubated.

	Specific activity	Relative fold
	nmol/min/mg	
Experiment 1		
R477, 30 °C	1.46	1
RL-25 (*firA200*), 30 °C	0.093	0.06
R477, 43 °C	1.46	1
RL-25 (*firA200*), 43 °C	0.046	0.03
Experiment 2		
BL21(DE3)/pLysS	1.36	1
BL21(DE3)/pLysS/pET11a	1.60	1.2
BL21(DE3)/pLysS/pTK106	486	357

Fig. 8 shows the reaction rate dependence of *N*-acylation on the concentrations of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN (*A*) and *R*-3-hydroxymyristoyl-ACP (*B*), using extracts of cells that overproduce the *firA*$^+$ protein. Values for K_m are estimated to be 1.3 μM for UDP-3-*O*-(*R*-3-hydroxymyristoyl)-

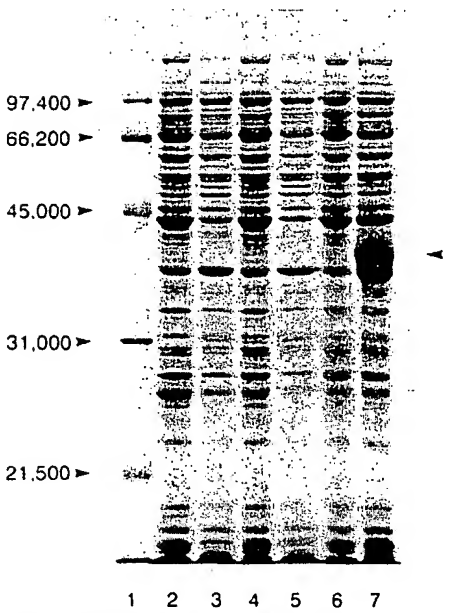

FIG. 7. **Overexpression of the *firA*⁺ gene product under the control of the T7 promoter.** Extracts of various strains were prepared as described under "Experimental Procedures." *Lane 1*, protein molecular weight standards; phosphorylase *b* (97,400); bovine serum albumin (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); and soybean trypsin inhibitor (21,500). *Lane 2*, strain BL21(DE3)/pLysS (host), before IPTG induction. *Lane 3*, strain BL21(DE3)/pLysS, after IPTG induction. *Lane 4*, strain BL21(DE3)/pLysS/pET11a (host/vector), before IPTG induction. *Lane 5*, strain BL21(DE3)/pLysS/pET11a, after IPTG induction. *Lane 6*, strain BL21(DE3)/pLysS/pTK106 (host/vector with *firA* insert), before IPTG induction. *Lane 7*, strain BL21(DE3)/pLysS/pTK106, after IPTG induction.

GlcN and 1.9 μM for *R*-3-hydroxymyristoyl-ACP. The UDP-GlcNAc *O*-acyltransferase has a K_m of 1.6 μM for *R*-3-hydroxymyristoyl-ACP (48).

Specificity of the N-acyltransferase for R-3-Hydroxymyristoyl-ACP—Myristoyl-ACP does not serve as an acyl donor for the overproduced UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase (Fig. 9), as is also the case for the purified UDP-GlcNAc *O*-acyltransferase (48). This finding is consistent with earlier studies of crude extracts of different Gram-negative bacteria, which indicated that the specificities of the *O*- and *N*-acyltransferases determine the characteristic hydroxy fatty acids attached to the glucosamine disaccharide of lipid A (27). Since only *R*-3-hydroxymyristate is found at the 2, 3, 2′, and 3′ positions of *E. coli* lipid A (1, 2), it is reassuring that both *E. coli* acyltransferases display extraordinary specificity for *R*-3-hydroxymyristoyl-ACP.

DISCUSSION

Mutations in the *firA* gene of *E. coli* were first isolated by Babinet in 1970 (12) and later characterized by Lathe *et al.* (28–30) in an attempt to identify components of the transcriptional apparatus other than RNA polymerase. They mapped the *firA200* allele near minute 4 on the *E. coli* chromosome and showed that it caused temperature-sensitive cell growth and reversed the high level of rifampicin resistance associated with mutations in the β subunit of RNA polymerase, encoded by the *rpoB* gene (hence, *fir* as opposed to *rif*) (28–30). RNA synthesis was also claimed to be thermosensitive in cells harboring *firA200*, but unequivocal evidence for selective inhibition of RNA synthesis under nonpermissive conditions

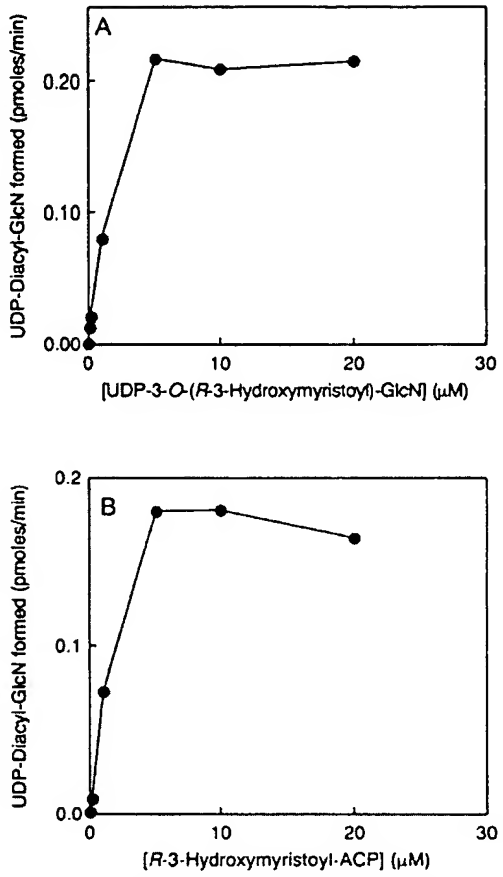

FIG. 8. ***N*-Acyltransferase reaction rate as a function of substrate concentrations.** The assays were carried out as described under "Experimental Procedures" with an extract of IPTG induced BL21(DE3)/pLysS/pTK106. *A*, the concentration of *R*-3-hydroxymyristoyl-ACP was held constant at 20 μM, and the extract was 100 ng/ml. *B*, the concentration of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN was held constant at 20 μM, and the extract was 20 ng/ml.

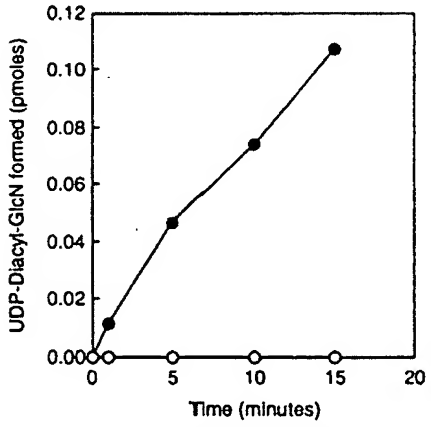

FIG. 9. **Myristoyl-ACP compared with *R*-3-hydroxymyristoyl-ACP as a substrate for the *N*-acyltransferase.** The assays were carried out as described under "Experimental Procedures" with an extract of IPTG-induced BL21(DE3)/pLysS/pTK106 (10 ng/ml). *Open circles*, myristoyl-ACP; *closed circles*, *R*-3-hydroxymyristoyl-ACP.

was not presented (28–30). Subsequent studies by Dicker and Seetharam (8, 11) demonstrated that the cloned *firA* gene encoded a 36-kDa polypeptide and that this gene was able to correct the phenotypes associated with the *firA200* mutation. Although the *firA* product is not a stoichiometric subunit of RNA polymerase, Dicker and Seetharam (8, 11) were able to demonstrate partial immunoprecipitation of the *firA* protein with RNA polymerase in strains that overproduced *firA*, but not in wild-type cells, using polyclonal antibodies directed against RNA polymerase (8). They suggested that *firA* might regulate the functioning of RNA polymerase, possibly by catalyzing some kind of covalent modification (8, 11). The important studies of Dicker and Seetharam (8, 11) also clarified earlier confusion regarding the relationship of *firA* and *hlpA*, both of which map near minute 4 of the *E. coli* chromosome (31, 32).

Vaara and co-workers (9, 10) independently identified a gene in *S. typhimurium*, termed *ssc*, that is highly homologous to *firA* (95.6% identity). Mutations in *ssc* render cells sensitive to antibiotics that normally do not penetrate the outer membrane. Further sequence analysis revealed low, but statistically significant, homology of both *firA* (8, 11) and *ssc* (9, 10) to *lpxA* (3, 7), an *E. coli* gene that encodes UDP-GlcNAc *O*-acyltransferase (3, 7, 19), the first enzyme of lipid A biosynthesis (Figs. 1 and 10). Interestingly, *firA* is located very close to *lpxA* (Fig. 11) on the chromosome (7, 8, 11). Both genes appear to be part of a complex operon encoding enzymes of lipid metabolism and DNA replication (7, 8, 24, 33, 34). Antibiotic hypersensitivity resembling that imparted by the *ssc* mutation is also observed in cells harboring *lpxA2* (13).

We have now demonstrated unequivocally that the product of the *firA* gene in *E. coli* has an enzymatic function in lipid A biosynthesis (Fig. 1). By using both a *firA* mutant strain (RL-25, *firA200*) and a strain that overproduces the *firA* gene product (BL21(DE3)/pLysS/pTK106), we have shown that *firA* encodes UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase (Fig. 1). Cells of strain RL-25 (*firA200*) display a significantly reduced rate of lipid A biosynthesis at the nonpermissive temperature (Fig. 2). With a newly developed direct assay for the third enzyme of the pathway (Figs. 1, 5, and 6), we found that extracts of the mutant strain are strikingly defective in UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase (Table III). Conversely, overproduction of the wild-type *firA* gene product, using the T7 expression system of Studier (15), gives rise to a 360-fold increase in UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase specific activity in cell extracts compared with wild-type (Table III). Given these findings, we suggest that *firA* be renamed *lpxD* (Figs. 1 and 11).

The fact that both the *lpxA* and the *firA* gene products utilize the same substrate (*R*-3-hydroxymyristoyl-ACP) to acylate adjacent positions on the glucosamine backbone of UDP-GlcNAc must account for the sequence similarity of these genes (Fig. 10). Careful sequence comparison, assisted by site-directed mutagenesis, should shed light on the important catalytic residues of these proteins. In this regard, there are two additional acyltransferases that play a role in later stages of lipid A biosynthesis in *E. coli* (35). These enzymes transfer laurate and/or myristate from lauroyl-ACP and/or myristoyl-ACP to the 2′ and 3′ *R*-3-hydroxymyristoyl groups of the intermediate (3-deoxy-D-*manno*-octulosonate)$_2$-lipid IV$_A$ (35). The genes encoding these enzymatic activities are not yet known, but the sequences of *lpxA* and *firA* may prove helpful for their discovery.

Myristoyl-ACP could not substitute for *R*-3-hydroxymyristoyl-ACP as the acyl donor in the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcN *N*-acyltransferase reaction (Fig. 9). UDP-GlcNAc *O*-acyltransferase displays a similar absolute specific-

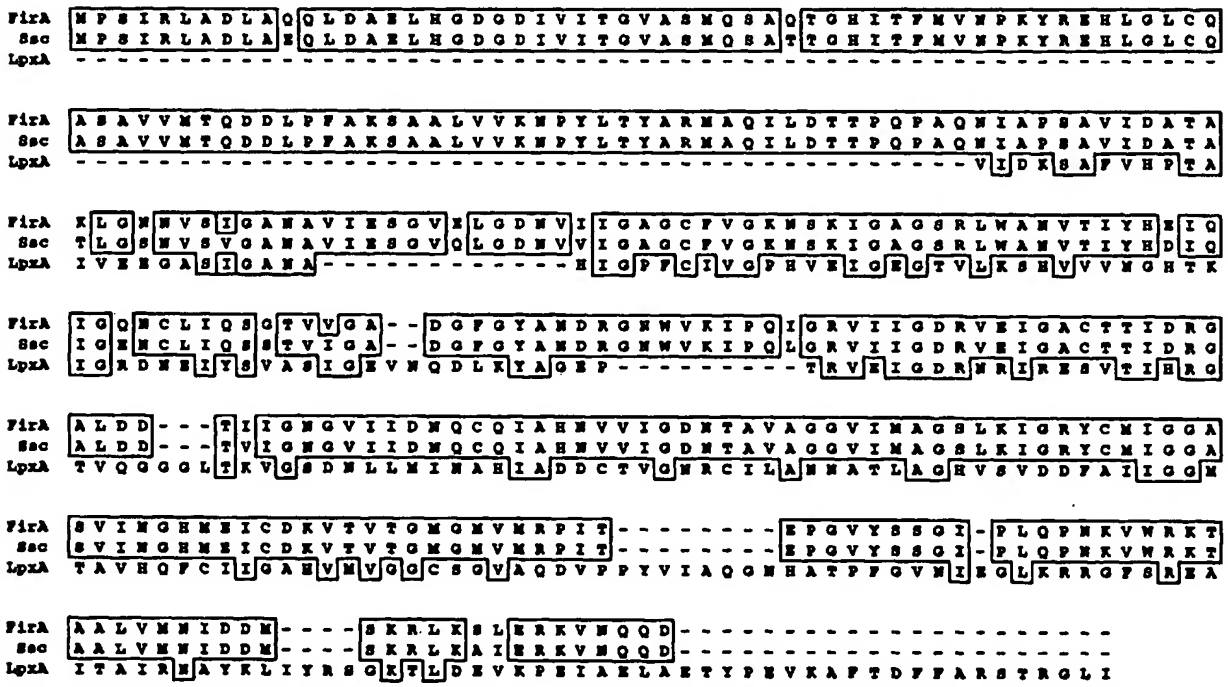

FIG. 10. **Similarity of the protein sequences of *firA*, *ssc*, and *lpxA*.** Over 260 residues of *firA* and *lpxA*, there is 27.6% identity and 49.3% similarity, with seven gaps. The quality of the sequence similarity between *lpxA* and *firA* is 7.7 standard deviations away from that obtained by randomization.

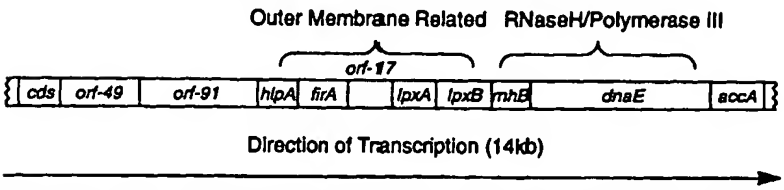

FIG. 11. **Organization of the genes in the vicinity of minute 4 on the *E. coli* chromosome.** The following functions have been identified: *cds*, CDP-diacylglycerol synthase (47); *hlpA*, basic outer membrane protein associated with lipopolysaccharide (8): *firA*, *N*-acyltransferase of lipid A biosynthesis; *lpxA*, UDP-GlcNAc *O*-acyltransferase (3, 19); *lpxB*, lipid A disaccharide synthase (3); *rnhB*, RNase H (32); *dnaE*, DNA polymerase III (33); *accA*, acetyl-coenzyme A carboxylase (24). Locations of promoters are not fully characterized.

ity for *R*-3-hydroxymyristoyl-ACP (22, 26, 48). Both acyltransferases from *E. coli* function poorly with longer or shorter fatty acyl moieties, and coenzyme A cannot substitute for ACP (26, 27). However, in *Pseudomonas aeruginosa* extracts, *N*-acylation is optimal with *R*-3-hydroxylauroyl-ACP (27). In *Rhizobium leguminosarum*, 14, 16, 18, or 28 carbon acyl moieties are found to be *N*-linked in lipid A (36) raising the possibility that multiple *N*-acyltransferases may exist. Peculiar *N*-linked fatty acyl moieties are also found on GlcNAc residues of nodulation factors produced by strains of *Rhizobium* (37, 38). In *Rhodobacter sphaeroides*, both *R*-3-hydroxymyristoyl and 3-ketomyristoyl residues are *N*-linked to the glucosamine disaccharide backbone of lipid A (39).

Lipid A has not been found in organisms other than Gram-negative bacteria (1, 2). However, several specific *N*-acyltransferases have been reported in eucaryotic cells, including ceramide synthase (40) and protein *N*-myristoyl transferase (41–43). The best studied of these is the protein *N*-myristoyl transferase that is responsible for the cotranslational amino-terminal myristoylation required for the functioning of some intracellular proteins involved in eucaryotic signal transduction (41–43). Protein *N*-myristoyl transferase is highly specific for hydrocarbon chain length, recognizes specific sequences at the amino termini of certain proteins, and utilizes coenzyme A thioesters as substrates (41–43). The protein *N*-myristoyl transferase does not have any obvious sequence homology to the *firA* gene product (data not shown), but it will be interesting to compare the three-dimensional structures of these enzymes in order to ascertain how they measure hydrocarbon chain length with such remarkable accuracy.

Although protein *N*-myristoyl transferase is absent in *E. coli* (44), protein *N*-acylation has been described in prokaryotes (45–47). For instance, the amino terminus of the major outer membrane lipoprotein of *E. coli* is acylated with palmitate, which is derived by transacylation from the 1 position of certain glycerophospholipids (46, 47). The possible *N*-acylation of proteins with *R*-3-hydroxymyristate has not been explored in *E. coli* or any other system. The intriguing idea that the *firA* gene product might be capable of acylating additional substrates, including proteins, deserves further exploration. If the *N*-acyltransferase actually were to catalyze the modification of selected proteins, such as the *rpoB* gene product, then a plausible mechanism for its participation in the regulation of RNA polymerase might yet be envisioned.

REFERENCES

1. Morrison, D. C., and Ryan, J. L. (eds) (1992) *Bacterial Endotoxic Lipopolysaccharides: Molecular Biochemistry and Cellular Biology*, Vol. I, CRC Press, Inc., Boca Raton, FL
2. Raetz, C. R. H. (1990) *Annu. Rev. Biochem.* **59**, 129–170
3. Crowell, D. N., Anderson, M. S., and Raetz, C. R. H. (1986) *J. Bacteriol.* **168**, 152–159
4. Young, K., Silver, L. L., Bramhill, D., Caceres, C. A., Stachula, S. A., Shelly, S. E., Raetz, C.R. H., and Anderson, M. S. (1993) *Fed. Proc.* **7**, 1252
5. Clementz, T., and Raetz, C. R. H. (1991) *J. Biol. Chem.* **266**, 9687–9696
6. Belunis, C. J., and Raetz, C. R. H. (1992) *J. Biol. Chem.* **267**, 9988–9997
7. Coleman, J., and Raetz, C. R. H. (1988) *J. Bacteriol.* **170**, 1268–1274
8. Dicker, I. B., and Seetharam, S. (1991) *J. Bacteriol.* **173**, 334–344
9. Vuorio, R., Hirvas, L., and Vaara, M. (1991) *FEBS Lett.* **292**, 90–94
10. Vaara, M. (1992) *FEMS Microbiol. Lett.* **97**, 249–254
11. Dicker, I. B., and Seetharam, S. (1992) *Mol. Microbiol.* **6**, 817–823
12. Babinet, C. (1970) *First Intern. Lepetit Coll.* 37–45
13. Vuorio, R., and Vaara, M. (1992) *Antimicrob. Agents Chemother.* **36**, 826–829
14. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
15. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89
16. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557–580
17. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp. 368–369, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Laemmli, U. K. (1970) *Nature* **227**, 680–685
19. Galloway, S. M., and Raetz, C. R. H. (1990) *J. Biol. Chem.* **265**, 6394–6402
20. Rock, C. O., and Cronan, J. E., Jr. (1980) *Anal. Biochem.* **102**, 362–364
21. Rock, C. O., and Cronan, J. E., Jr. (1979) *J. Biol. Chem.* **254**, 7116–7122
22. Anderson, M. S., Bulawa, C. E., and Raetz, C. R. H. (1985) *J. Biol. Chem.* **260**, 15536–15541
23. Japanese Patent Publication JP61205229-A (September 11, 1986)
24. Li, S.-J., and Cronan, J. E., Jr. (1992) *J. Biol. Chem.* **267**, 16841–16847
25. Anderson, M. S., Robertson, A. D., Macher, I., and Raetz, C. R. H. (1988) *Biochemistry* **27**, 1908–1917
26. Anderson, M. S., and Raetz, C. R. H. (1987) *J. Biol. Chem.* **262**, 5159–5169
27. Williamson, J. M., Anderson, M. S., and Raetz, C. R. H. (1991) *J. Bacteriol.* **173**, 3591–3596
28. Lathe, R., Lecocq, J. P., and Resibois, A. (1977) *Mol. & Gen. Genet.* **154**, 43–51
29. Lathe, R., and Lecocq, J. P. (1977) *Mol. & Gen. Genet.* **154**, 53–60
30. Lathe, R. (1977) *J. Bacteriol.* **131**, 1033–1036
31. Aasland, R., Coleman, J., Holck, A. L., Smith, C. L., Raetz, C. R. H., and Kleppe, K. (1988) *J. Bacteriol.* **170**, 5916–5918
32. Hirvas, L., Coleman, J., Koski, P., and Vaara, M. (1990) *FEBS Lett.* **262**, 123–126
33. Tomasiewicz, H. G., and McHenry, C. S. (1987) *J. Bacteriol.* **169**, 5735–5744
34. Itaya, M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8587–8591
35. Brozek, K. A., and Raetz, C. R. H. (1990) *J. Biol. Chem.* **265**, 15410–15417
36. Hollingsworth, R. I., and Carlson, R. W. (1989) *J. Biol. Chem.* **264**, 9300–9303
37. Fisher, R. F., and Long, S. R. (1992) *Nature* **357**, 655–660
38. Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. (1991) *Nature* **354**, 125–130
39. Qureshi, N., Honovich, J. P., Hara, H., Cotter, R. J., and Takayama, K. (1988) *J. Biol. Chem.* **263**, 5502–5504
40. Kishimoto, Y. (1983) in *The Enzymes* (Boyer, P. D., ed) Vol. XVI, pp. 357–407, Academic Press, Orlando, FL
41. Duronio, R. J., Rudnick, D. A., Johnson, R. L., Johnson, D. R., and Gordon, J. I. (1991) *J. Cell Biol.* **113**, 1313–1330
42. Knoll, L. J., Levy, M. A., Stahl, P. D., and Gordon, J. I. (1992) *J. Biol. Chem.* **267**, 5366–5373
43. Rudnick, D. A., Johnson, R. L., and Gordon, J. I. (1992) *J. Biol. Chem.* **267**, 23852–23861
44. Duronio, R. J., Jackson-Machelski, E., Hueckeroth, R. O., Olins, P. O., Devine, C. S., Yonemoto, W., Slice, L. W., Taylor, S. S., and Gordon, J. I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1506–1510
45. Braun, V., and Rehn, K. (1969) *Eur. J. Biochem.* **10**, 426–438
46. Gupta, S. D., Dowhan, W., and Wu, H. C. (1991) *J. Biol. Chem.* **266**, 9983–9986
47. Raetz, C. R. H. (1986) *Annu. Rev. Genet.* **20**, 253–295
48. Anderson, M. S., Bull, H. G., Galloway, S. M., Kelly, K., Mohan, S., Radika, K., and Raetz, C. R. H. (1993) *J. Biol. Chem.* **268**, 19858–19865